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Award Number: W81XWH-04-1-0286

TITLE: The Role of the Sonic Hedgehog Pathway for Prostate Cancer Progression

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REPORT DATE: February 2007

TYPE OF REPORT: Final

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

# REPORT DOCUMENTATION PAGE

*Form Approved*
*OMB No. 0704-0188*



| 1. REPORT DATE | 2. REPORT TYPE | 3. DATES COVERED |
|---|---|---|
| 01-02-2007 | Final | 30 Jan 2004– 29 Jan 2007 |

**4. TITLE AND SUBTITLE**

The Role of the Sonic Hedgehog Pathway for Prostate Cancer Progression

**5a. CONTRACT NUMBER**

**5b. GRANT NUMBER**
W81XWH-04-1-0286

**5c. PROGRAM ELEMENT NUMBER**

**6. AUTHOR(S)**

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**5d. PROJECT NUMBER**

**5e. TASK NUMBER**

**5f. WORK UNIT NUMBER**

**7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)**

University of Texas Medical Branch
Galveston, TX 77555-1048

**8. PERFORMING ORGANIZATION REPORT NUMBER**

**9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)**
U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

**10. SPONSOR/MONITOR'S ACRONYM(S)**

**11. SPONSOR/MONITOR'S REPORT NUMBER(S)**

**12. DISTRIBUTION / AVAILABILITY STATEMENT**
Approved for Public Release; Distribution Unlimited

**13. SUPPLEMENTARY NOTES**
Original contains colored plates: ALL DTIC reproductions will be in black and white.

**14. ABSTRACT**

The hedgehog pathway plays a critical role in the development of prostate. However, the role of the hedgehog pathway in prostate cancer is not clear. In the previous funding period, we have reported activation of hedgehog signaling in advanced and metastatic tumors. We also reported one possible molecular mechanism by which activated hedgehog signaling alter cell functions. Here, we report another mechanism by which hedgehog signaling is activated in prostate cancer. We found that the Shh promoter activity is elevated in several cancer cell lines with hedgehog signaling activation, including LNCaP, Huh7 cells. Growth of these cells depends on the presence of ligand, sonic hedgehog. Following treatment of neutralizing antibodies to sonic hedgehog, we observed reduced cell growth and apoptosis in several cancer cell lines. Through further analyses, we narrowed the region responsible for sonic hedgehog promoter activity to a region less than 1kb. Currently, we are trying to identify the transcriptional factors involved in regulation of sonic hedgehog expression. Furthermore, we have made some progress in animal models for hedgehog signaling-mediated carcinogenesis. Currently, we have established conditioned knockout of Ptch1 (or knock/in of activated SMO) in mice using Keratin 14 promoter, which targets not only skin epidermis but also prostate epithelium. These mice will be mated with other genetically engineered mice to obtain bigenic mice in our analysis of prostate carcinogenesis.

**15. SUBJECT TERMS**
Prostate cancer, Suppressor of fused, hedgehog, Patched

| 16. SECURITY CLASSIFICATION OF: | | | 17. LIMITATION OF ABSTRACT | 18. NUMBER OF PAGES | 19a. NAME OF RESPONSIBLE PERSON |
|---|---|---|---|---|---|
| a. REPORT | b. ABSTRACT | c. THIS PAGE | | | 19b. TELEPHONE NUMBER *(include area code)* |
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**Standard Form 298 (Rev. 8-98)**
**Prescribed by ANSI Std. Z39.18**

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**Introduction**

This proposal is to evaluate the role of the hedgehog pathway in prostate cancer in clinical specimens, and to identify the molecular basis of hedgehog mediated tumor formation.

**Body**

Good progress has been made on this project in the third year. Two manuscripts directed associated with this proposal have been published. ***First***, we revealed a new mechanism by which hedgehog signaling is activated in human cancer, including prostate and hepatocellular carcinomas: through transcriptional activation of the ligand Shh (Carcinogenesis 27: 1334-40, 2006). ***Second***, our studies of Su(Fu) inactivation in lung cancer has now been published in Cancer Letters (244: 53-60, 2006), and we believe that this mechanism exists in other types of human cancers, including prostate cancer. ***Third,*** we have started to generate conditional activation of hedgehog signaling in the prostate. These mice will be crossed to other genetically engineered mice (such as nkx3.1 null mice). Due to the time required for mouse mating and tumorigenesis, we anticipate a long time before a phenotype can be observed. We have requested one year no-cost extension. In summary, our findings are very important for our understanding of hedgehog-mediated prostate cancer development.

**Task 1:** (completed) **Task 2:** (completed last year) **Task 3**: (partly completed, see below)

Because nearly 50% of Ptch1+/- mice die of medulloblastomas or rhabdomyosarcomas, we have established two systems for activated hedgehog signaling in the prostate. In the first system, we established keratin 14 promoter-driven Ptch1 knockout, which will allow activation of hedgehog signaling in prostate as well as other keratin 14 expressing epithelial tissues. In the second system, we expressed activated SMO molecule, SMO-m2, in a tissue specific manner also under the control of keratin 14 promoter. In the last six months, we have examined over 20 mice with activation of hedgehog signaling in both systems, but did not find any significant changes in prostate growth and formation of prostate hyperplasia in mice less than 4 month old. **Fig. 1** shows our genotyping of allelic recombination of the Ptch1 locus. There might be many reasons for this result. First, development of prostate cancer occurs often in older mice (even in the PTEN knockout mice). Thus, we may find hyperplasia or tumor in the prostate in the old animals. To that end, we will continue to monitor some of the old mice for changes in the prostate. Second, since activation of the hedgehog pathway is frequently found in advanced and metastatic cancers of prostate. It is possible that hedgehog signaling plays an important role in tumor metastasis. To explore that possibility, we have talked with Professor Ping Wu from UCLA to collaborate using mouse model of prostate cancer using PTEN knockout mice. Eventually, this mouse model will help us to understand hedgehog signaling for development of prostate cancer. Furthermore, Professor Leland Chung has started collaboration with my laboratory on the effects of hedgehog signaling for prostate cancer metastasis. All these long-term studies are necessary for us to design novel therapeutic approaches to treat prostate cancer.

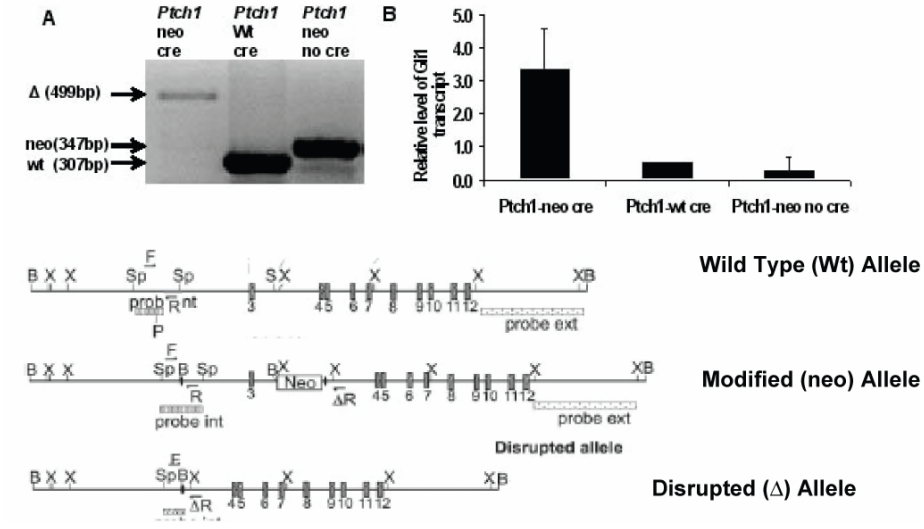


**Fig 1:** Genetic recombination occurred in $Ptc^{floxp}$ keratinocytes following expression of Keratin 14 promoter driven cre to generate a 499 bp PCR product (**A**); wild-type cells expressing K14- or $Ptc^{floxp}$ keratinocytes without cre expression do not yield this PCR product. Genetically Ptch1 knockdown cells were expected to have activated Hh signaling and therefore increased Gli1 expression (**B**). This approach can be used to generate prostate-specific Ptch1 knockdown to study the role of Hh signaling in prostate cancer. Wild-type, modified and disrupted Ptch1 allele were described previously..

In addition to these tasks, we have discovered another mechanism by which hedgehog signaling is activated in human cancer, including prostate cancer. We found that sonic hedgehog, the ligand of hedgehog pathway, is frequently up-regulated in prostate cancer cells. **Fig. 2** shows a luciferase reporter gene analysis using sonic hedgehog promoter construct in LNCaP cells. In addition to prostate cancer, we also found activated sonic hedgehog promoter activity in hepatocellular carcinomas. **Fig. 3** shows that the sonic hedgehog promoter activity is high in Huh7 cells but low in HepG2 cells. In the presence of HCV replicon, we detected an additional increase in the sonic hedgehog promoter activity, suggesting that HCV somehow activates the sonic hedgehog

promoter activity. This analysis is consistent with our data on the expression of sonic hedgehog transcript and hedgehog target gene Gli1 (**Fig. 4**). Furthermore, we have shown that elevated expression of sonic hedgehog is functionally relevant to cancer cell growth (**Fig. 5**). All these data indicate that elevated expression of sonic hedgehog is an important mechanism by which the hedgehog pathway is activated in human cancer, including hepatocellular carcinomas and prostate cancer.

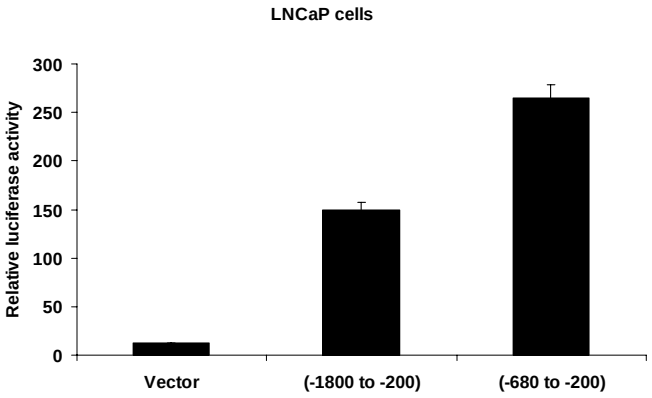


**Fig. 2 Analysis of Shh promoter reporter activity in prostate cancer cells.** We cloned human two sonic hedgehog promoter fragments (-1,800 to -200 and -680 to -200) into the luciferase reporter plasmid pGL4.14. 48 hr following transfection of the sonic hedgehog promoter constructs or the control vector (together with renilla luciferase reporter plasmid as the transfection control), we performed dual luciferase analyses. Consistent with elevated expression of sonic hedgehog in our previous study (Sheng et al, 2004, Molecular Cancer 3, 29), we found a high sonic hedgehog promoter activity in LNCaP cells. We found that a short fragment of sonic hedgehog promoter region (-680 to -200) retains a high level of promoter activity.

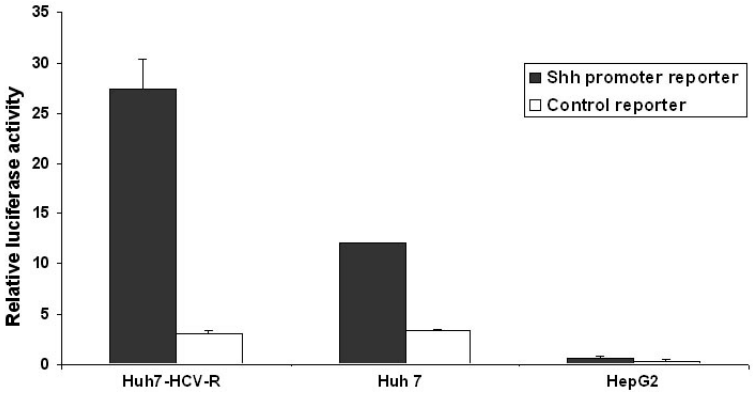


**Fig. 3 Different levels of Shh promoter activity in different HCC cell lines.** The reporter activity was measured 48 hours post-transfection of the plasmids (the Shh promoter reporter or the control pGL4.14, renilla luciferase reporter pGL4.70) into HCC cells. Huh7-HCV-R cells are derived from Huh7 cells, containing HCV replicons. The Shh promoter activity in Huh7 and HepG2 cells is consistent with the level of Shh transcript (see Figure 2E for comparison). In the presence of HCV replicons, we observed an increase in the Shh reporter activity. We concluded from these data that increased expression of Shh in HCC resulted from up-regulation of Shh transcription, which can be regulated by HCV replication.

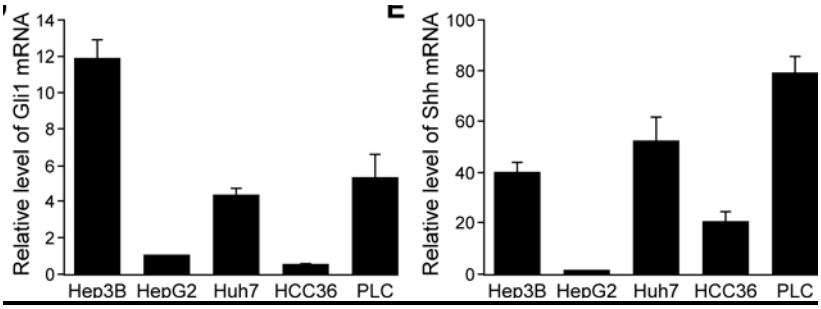


**Fig. 4 Real-time PCR analysis of Gli1 and sonic hedgehog transcripts.** Real-time PCR was performed as previously reported (Huang et al, 2006). We found a relatively high level of *Gli1* and sonic hedgehog in Hep3B, Huh7 and PLC cells. Data indicates values relative to 18S RNA and to a calibrator. Elevated sonic expression is associated with a high level of hedgehog target gene expression, suggesting that sonic hedgehog expression may be responsible for activation of the hedgehog pathway.

## Key Research Accomplishments

With support from DOD, we have revealed a novel mechanism by which the hedgehog signaling pathway is activated in human cancer, including prostate cancer and hepatocellular carcinomas (***Carcinogenesis*** 27:1334-40, 2006). We have made progress in mouse models of hedgehog signaling activation in the prostate. To complete our research task 3, we have requested a one year no-cost extension to examine any effects of hedgehog signaling in prostate cancer development.

## Reportable outcomes

Four research papers (***Carcinogenesis*** 27:1334-40, 2006; ***Cancer Letters*** 244: 53-60, 2006)

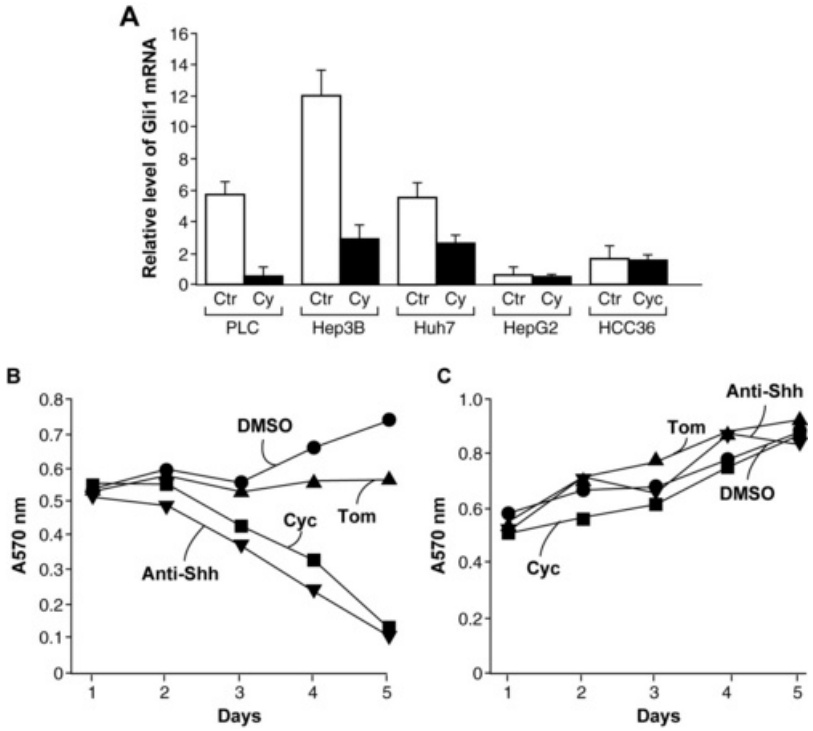


**Figure 5 Hedgehog signaling and Growth of HCC cells.** Real-time PCR data of *Gli1* transcript shows that in the presence of 2μM hedgehog inhibitor cyclopamine (**A**) or 1μg/ml sonic hedgehog neutralizing antibodies (data not shown here) for 12 hours, the level of hedgehog target gene Gli1 was decreased in the three cell lines with activated hedgehog signaling (PLC, Hep3B and Huh7). In contrast, no effects were observed in HCC36 and HepG2 cells, in which hedgehog signaling is not activated. Cell growth of Huh7 (**B**) and HepG2 (**C**) cell lines were examined by MTT assay. Huh7 cells were inhibited by 2 M cyclopamine (Cat# K317000, Toronto Research Chemicals, Canada) or 1 g/ml Shh neutralizing antibodies (Cat# 9024, Santa Cruz Biotechnology Inc) (**B**). This inhibition was specific because addition of tomatidine, a structurally similar but non-specific compound for hedgehog signaling did not affect cell growth. In contrast, cell growth of HepG2 was not affected by cyclopamine (2μM) or Shh neutralizing antibodies (1 μg/ml) (**C**), confirming the specific growth inhibition of HCC cells through targeted inactivation of hedgehog signaling.

## Conclusion

We have demonstrated that hedgehog pathway activation occurs frequently in advanced prostate cancer, lung cancer and hepatocellular carcinomas. One mechanism is through inactivation of negative regulator Su(Fu), and the other mechanism is through elevated expression of sonic hedgehog. Long-term collaborations have been established to investigate hedgehog signaling for development of prostate cancer.

## References

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**2.** Huang S, He J, Zhang X, Bian Y, Yang L, Xie G, Zhang K, Tang W, Stelter AA, Wang Q, Zhang H, Xie J. Activation of the hedgehog pathway in human hepatocellular carcinomas. Carcinogenesis 27(:1334-40, 2006.

## Appendices

1. Reprint of Carcinogenesis, 2006.
2. Reprint of Cancer Letters, 2006.

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Cancer Letters 244 (2006) 53–60
CANCER Letters
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# Activation of the hedgehog pathway in a subset of lung cancers

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Received 12 July 2005; received in revised form 19 October 2005; accepted 28 November 2005

**Abstract**

Activation of the hedgehog pathway is reported in lung cancer, but its frequency remains unknown. We examine activation of this pathway in lung cancers by in situ hybridization and immunohistochemstry, and find that less than 10% of the tumors have elevated hedgehog target gene expression. We further identify a cell line NCI-H209 and two primary tumors with no detectable Su(Fu), a negative regulator of the pathway. Ectopic expression of Su(Fu) in NCI-H209 cells down-regulates hedgehog target gene expression and leads to inhibition of cell proliferation. These data indicate that activation of the hedgehog pathway is activated through Shh over-expression or Su(Fu) inactivation in only a subset of lung cancers.


*Keywords:* Hedgehog; Lung cancer; Gli1; Su(Fu); PTCH1

## 1. Introduction

The hedgehog pathway plays a critical role in embryonic development and tissue formation, including foregut [1]. Targeted deletions of sonic hedgehog, Gli2 or Gli3 result in foregut malformation and embryo lethality in mice [2–4]. Secreted Hh molecules bind to the receptor patched (PTC-PTCH1, PTCH2), thereby alleviating PTC-mediated suppression of smoothened (SMO), a putative seven-transmembrane protein. SMO signaling triggers a cascade of intracellular events, leading to activation of the pathway through GLI-dependent transcription [5,6]. Activation of Hh signaling, through loss-of-function mutations of PTCH1 or activated mutations of SMO, occurs frequently in human basal cell carcinomas (BCCs) and medulloblastomas [7–16]. More recently, abnormal activation of the sonic hedgehog pathway has been reported in

*Abbreviations* PTC, patched; PTCH1, human patched gene 1; NSCLC, non-small cell lung cancer; shh, sonic hedgehog; BCC, basal cell carcinoma; Su(Fu), suppressor of fused.

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doi:10.1016/j.canlet.2005.11.036

subsets of small cell lung cancer, pancreatic cancer, prostate cancer, and gastrointestinal (GI) cancers [17–23].

Lung cancer is the leading cause of cancer-related death, claiming more than 150,000 lives every year in the US alone (which exceeds the combined mortality from breast, prostate, and colorectal cancers). Patients with advanced stage of lung cancer, which represents 75% of all new cases, have a median survival time of only 10 months. Thus, identifying an effective biomarker for early diagnosis of lung cancer is the first essential step to reduce the mortality. Activation of hedgehog signaling was reported in five of 10 small cell lung cancers and four of 40 non-small cell lung cancers (NSCLC) [17]. To determine if hedgehog signaling activation can be utilized for diagnosis and treatment of lung cancer, we performed a comprehensive study to assess hedgehog pathway activation in specimens from 172 lung cancer patients and five patients without lung cancer by in situ hybridization and immunohistochemistry.

## 2. Materials and methods

### 2.1. Patient material

A total of 177 patients (172 lung cancer patients and five patients without cancer) were included in our study with approval of Institutional Research Board. Specimens from 96 patients were received as discarded materials from University of Texas Medical Branch Surgical Pathology and the Shan Dong Qi Lu Hospital, Jinan, China. Lung cancers and the matched lung tissues were collected from each patient whenever possible. For tumors without matched normal tissues, a portion of lung tissue surrounding the tumor was used. Pathology reports and H&E stained slides from each specimen were reviewed to determine the nature of the disease and the tumor histology [24]. The randomly sorted samples with masked identity were evaluated by at least two independent certified pathologists. Lung cancers were divided into the following subtypes: adenocarcinoma, squamous cell carcinoma, alveolar cell carcinoma, adenosquamous cell carcinomas, large cell carcinoma, small cell carcinoma and carcinoid. For tissue microarray, we have triplicates for each specimen [25]. Both tumor tissues and the matched normal tissues (or the surrounding tissues) were included in our study.

In addition, we purchased a tissue microarray of lung cancer from Chaoying Biotechnology Co. Ltd (Xi'an, China), which contains 81 informative specimens (including five noncancerous lung tissues as controls). Analyzes of these specimens were described in each experimental method.

### 2.2. In situ hybridization

Using probes for Gli1, PTCH1 and HIP, *in situ* hybridization was performed in specimens listed in Supplementary Table 1 according to our previously published protocol [26,27]. Matched normal lung tissues or tissues surrounding tumors were also included in the study. Sense and antisense probes were obtained by T3 and T7 in vitro transcription using a kit from Roche (Mannheim, Germany). Blue indicated strong hybridization. As negative controls, sense probes were used in all hybridization and no positive signals were observed.

### 2.3. RNA isolation, quantitative PCR and northern blotting

Total RNAs were extracted using a RNA extraction kit from Promega according to the manufacturer (Promega, Madison, WI). Real-time PCR analyzes were performed according to Ma et al. [26,27]. Northern blotting was performed as previously reported [28].

### 2.4. Immunohistochemistry

Representative formalin-fixed and paraffin embedded tissue sections (6 μm thickness) were used for immunohistochemistry with specific antibodies to human Shh, PTCH1, Su(Fu) and HIP [Cat. No. 9024 for Shh and Cat. No. 6149 for PTCH1, Cat. No. 10934 for Su(Fu), Santa Cruz Biotechnology, Inc.; Cat. No. AF1568 for HIP antibodies, R&D Systems, Inc.]. All primary antibodies have been previously tested for immunohistostaining [22,23]. Immunohistochemistry of PTCH1 and Shh was carried out as previously reported [22,29] on specimens listed in Supplementary Tables 1 and 2. HIP protein expression was also detected by immunohistochemistry in the specimens listed in Supplementary Table 2. Detection of Su(Fu) protein was only performed in several specimens with activated hedgehog signaling.

### 2.5. Cell culture, colony formation assay, BrdU labeling and MTT assay

Human lung cancer cell lines (A549, H82, H187, H196, H209, H460, H661, H1299, BEAS2-B and BZR-T33) were purchased from ATCC and cultured in the recommended media from ATCC [28]. Expression of Su(Fu), under the control of the CMV promoter, in NCI-H209 cells was achieved by retrovirus-mediated gene transfer [29]. BrdU labeling was performed as previously described [29]. Flow cytometry was performed in our core facility [28]. Colorimetric MTT assay was performed according to our published protocol in the presence of 0.5% FBS [30,31].

Student's *t*-test for two samples was performed for the difference between tumor groups: $P < 0.05$ was considered statistically significant.

## 3. Results

To assess the frequency of hedgehog signaling activation in primary lung cancers, we initially examined expression of hedgehog target genes Gli1 and PTCH1 in 81 cases of lung specimens in a tissue microarray (see Supplementary Table 1 for specimen information). Increased levels of both PTCH1 and Gli1 transcripts indicate activation of the hedgehog pathway [5].

We first detected Gli1 and PTCH1 transcripts using in situ hybridization. In agreement with a published report [17], we did not detect Gli1 and PTCH1 in normal lung tissues, suggesting that the hedgehog pathway is not normally activated in adult normal lung tissues (Fig. 1A and A′). In contrast, we detected expression of both Gli1 and PTCH1 transcripts in 8 of 76 tumor specimens (Table 1; Fig. 1B, C, B′ and C′), suggesting that activation of the hedgehog pathway occurs in a subset of lung cancers. Further analyzes indicated that activation of the hedgehog pathway is not restricted to any specific subtypes of lung cancers (see Table 1, positive tumors include three adenocarcinomas, two squamous cell carcinomas, one small cell carcinoma, one large cell carcinoma and one alveolar cell carcinoma).

Expression of PTCH1 in lung cancer specimens (see Supplementary Table 1) was further confirmed by immunohistochemistry (Fig. 1E and F) [22,23]. All tissues with detectable PTCH1 protein had elevated PTCH1 transcript. With these data, we expanded the study to include additional 96 lung cancer specimens. Immunohistochemistry using antibodies identified additional eight tumors with detectable expression of both PTCH1 and HIP [22] (Fig. 1H; Table 2; Supplementary Table 2), indicating activation of the hedgehog pathway in 8 of 96 tumors. These data confirm that activation of the hedgehog pathway occurs only in a small subset of lung cancers.

In total, we found 15 out of 172 lung cancers (8.7%) harboring activated hedgehog signaling. Due to limited number of tumors with activated hedgehog signaling (detectable expression of at least two hedgehog target genes, PTCH1, Gli1 or HIP), it was not possible to perform statistical analysis. We also examined expression of PTCH1 protein by immunohistochemistry in lung cancer metastases (lymph node and intra-lung metastases) and identified 4 of 38 metastases of NSCLC

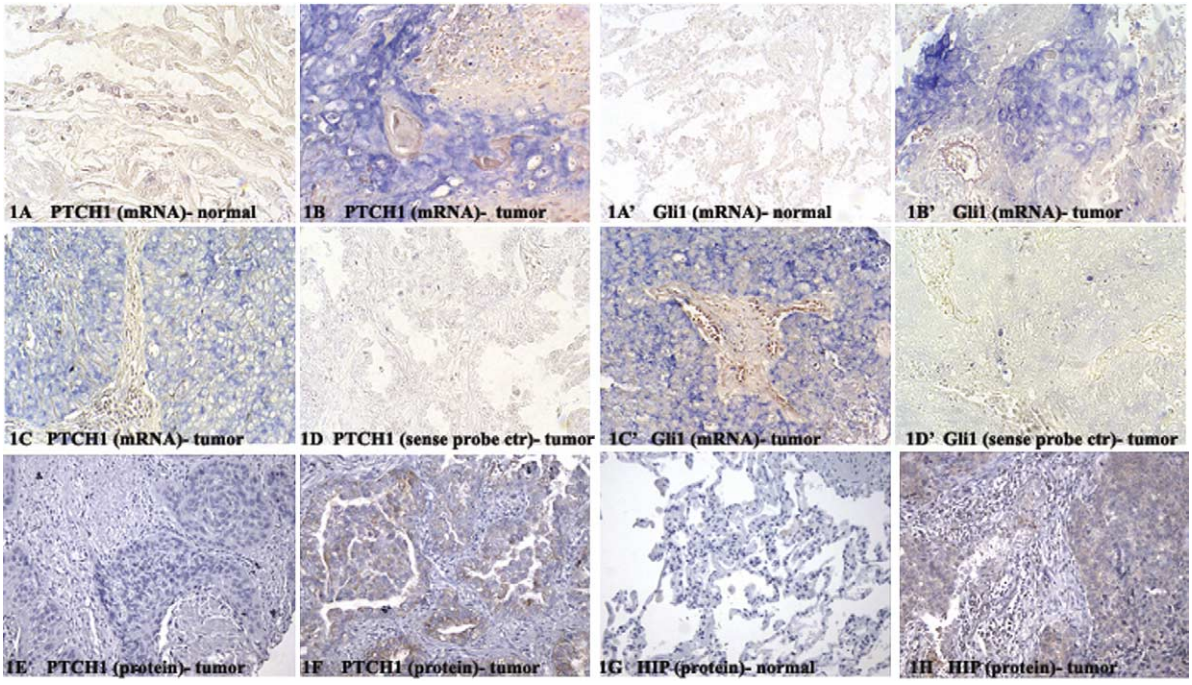


Fig. 1. Expression of hedgehog target genes in lung specimens. The levels of PTCH1 (1A–C), Gli1 (1A′–C′) transcripts (100×, blue as positive) were detected by in situ hybridization in normal and tumors (see Supplementary Table 1 for the list of specimens). The sense probe control did not give signal (see D and D′). The result was shown as '+++' for strong staining, as '++' for staining, as '+' for weak staining. Negative staining was shown as '−'. Protein expression of PTCH1 (CE and F), HIP (G and H) was detected by immunohistochemistry in all specimens with specific antibodies (see Section 2 for details, 100×) (brown–red as positive). The result was shown as '+++' for strong staining, as '++' for staining, as '+' for weak staining. Negative staining was shown as '−'. (For interpretation of the reference to colour in this legend, the reader is referred to the web version of this article).

Table 1
Expression of Shh, PTCH1 and Gli1 in lung cancer (in situ hybridization)

| | Tumor types | Total | Shh | | PTCH1/Gli1 | |
|---|---|---|---|---|---|---|
| | | | Positive | (%) | Positive | (%) |
| | | 76 | 64 | 84.2 | 8 | 10.5 |
| Subtypes | Adenocarcinoma | 27 | 21 | 80.8 | 3 | 11.1 |
| | Alveolar cell carcinoma | 9 | 7 | 77.8 | 1 | 11.1 |
| | Large cell carcinoma | 5 | 4 | 80.0 | 1 | 20.0 |
| | Small cell carcinoma | 10 | 8 | 80.0 | 1 | 10.0 |
| | Squamous cell carcinoma | 25 | 23 | 92.0 | 2 | 8.0 |
| | Carcinoid | 1 | 1 | | 0 | |
| Grade | Well differentiated | 5 | 5 | 100 | 0 | 0 |
| | Moderately differentiated | 18 | 16 | 88.9 | 2 | 11.1 |
| | Poorly differentiated | 26 | 22 | 84.6 | 2 | 7.7 |
| | UND[a] | 27 | 21 | 77.8 | 4 | 14.8 |
| Sex | Female | 19 | 15 | 78.9 | 1 | 5.2 |
| | Male | 57 | 49 | 85.9 | 7 | 12.3 |

[a] Information not available.

with PTCH1 positive staining, suggesting that activation of the hedgehog pathway is not specifically associated with lung tumor metastases. Expression of hedgehog targets resides to the tumor nest, not to the stroma, suggesting that hedgehog signaling is not very active in the stroma, which is quite different from other situations such as during lung development [1] or in gastric cancers [27].

Next, we tested expression of molecules potentially involving in hedgehog signaling activation. It is reported that Shh over-expression is responsible for activation of the hedgehog pathway in pancreatic cancer [23], gastric cancer [18,27] and lung cancer [17]. To test this possibility, we first examined expression of Shh in lung specimens by in situ hybridization in lung cancer specimens listed in Supplementary Table 1. As expected, Shh expression was undetectable in all three normal lung tissues examined (Fig. 2A). In contrast, many primary tumors expressed Shh (Fig. 2B; Tables 1 and 2). In agreement with the in situ hybridization data, we detected Shh protein in tumors with detectable Shh transcript (Fig. 2C

Table 2
Expression of Shh, PTCH1 and HIP in lung cancer (immunohistochemistry)

| | Tumor types | Total | Shh | | PTCH1/Gli1 | |
|---|---|---|---|---|---|---|
| | | | Positive | (%) | Positive | (%) |
| | | 96 | 63 | 65.6 | 8 | 8.3 |
| Subtype | Adenocarcinoma | 37 | 21 | 56.8 | 3 | 8.1 |
| | Alveolar cell carcinoma | 3 | 2 | | 0 | |
| | Large cell carcinoma | 5 | 4 | 80.0 | 1 | 20.0 |
| | Small cell carcinoma | 3 | 2 | | 0 | |
| | Squamous cell carcinoma | 42 | 31 | 73.8 | 4 | 9.5 |
| | Carcinoid | 2 | 1 | | 0 | |
| | Adenosquamous cell carcinoma | 4 | 2 | | 0 | |
| Grade | Well differentiated | 12 | 8 | 66.7 | 0 | 0 |
| | Moderately differentiated | 44 | 31 | 70.5 | 5 | 11.4 |
| | Poorly differentiated | 36 | 20 | 55.6 | 3 | 8.3 |
| | UND[a] | 3 | 3 | | 0 | |
| Stage | I | 48 | 29 | 60.4 | 1 | 2.1 |
| | II | 24 | 16 | 66.7 | 3 | 12.5 |
| | III/IV | 20 | 15 | 75.0 | 4 | 20.0 |
| | UND[a] | 4 | 3 | | 0 | |
| Sex | Female | 35 | 25 | 71.4 | 3 | 8.6 |
| | Male | 61 | 38 | 62.3 | 5 | 8.2 |

[a] Information not available.

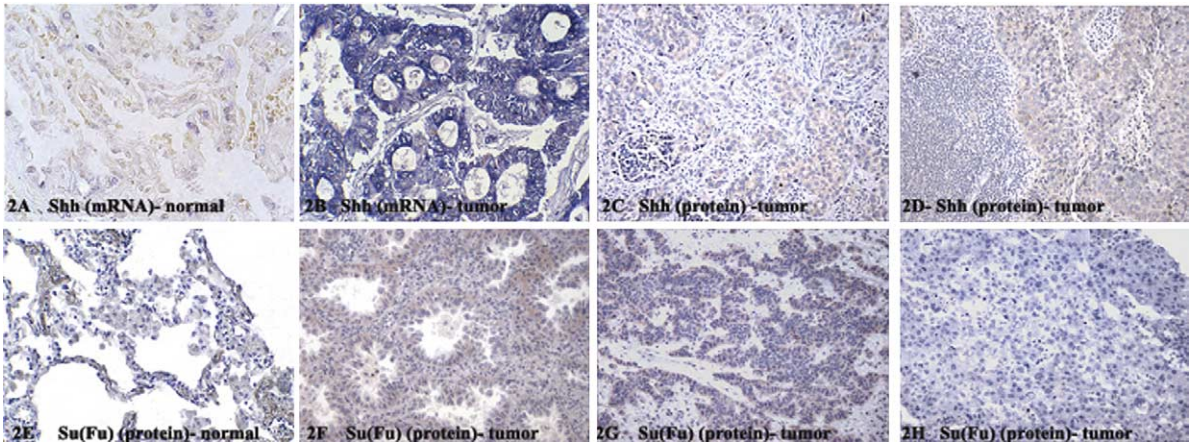


Fig. 2. Expression of Shh and Su(Fu) in lung specimens. The level of Shh transcript (A and B) was detected by in situ hybridization in the specimens listed in Supplementary Table 1 (100×, blue as positive). The proteins of Shh (C and D) and Su(Fu) (E–H) were assessed by immunohistochemistry (100×, Brown–red as positive). The result was shown as '+++' for strong staining, as '++' for staining, as '+' for weak staining. Negative staining was shown as '−'. (For interpretation of the reference to colour in this legend, the reader is referred to the web version of this article).

and D; Supplementary Table 1). Shh was detectable specifically in the tumor, not in the stroma (Fig. 2C and D), suggesting that that a paracrine signaling mechanism of sonic hedgehog does not play an important role in lung cancers. Furthermore, expression of Shh protein was also detected in specimens listed Supplementary Table 2. In all, over 73% of lung cancers had detectable Shh expression (Tables 1 and 2). We found that expression of Shh is not always associated with expression of hedgehog target genes in lung cancers ($P = 0.8444$). Furthermore, 5 of the 16 tumors, which have detectable expression of at least two hedgehog target genes, did not have detectable expression of Shh (Supplementary Tables 1 and 2), suggesting that over-expression of Shh may be partially responsible for activating hedgehog signaling pathway in lung cancers.

To identify additional molecular mechanisms for hedgehog signaling activation, we detected expression of other components of the hedgehog pathway, including Su(Fu), a negative regulator of hedgehog signaling [32,33]. Like PTCH1, loss of Su(Fu) is reported to be responsible for hedgehog signaling activation in subset of medulloblastomas [12], prostate cancer [22] and basal cell carcinomas [11]. We found that two tumors with elevated levels of PTCH1 and Gli1 had no detectable levels of Su(Fu), one of the tumors had no Shh expression (Fig. 2E–H and Supplementary Table 2), suggesting that loss of Su(Fu) may be also responsible for hedgehog signaling activation in a small number of lung cancers.

To substantiate our findings in the tumors, we examined eight lung cancer cell lines for Su(Fu) expression and found one cell line NCI-H209 with no detectable Su(Fu) protein (Fig. 3A and Supplementary Fig. 1). Southern hybridization using Su(Fu) probe did not reveal dramatic genomic changes of the Su(Fu) gene in H209 cells (data not shown here). Northern analysis showed no detectable Su(Fu) transcript in NCI-H209 cells (Fig. 3A), indicating a possible transcriptional silencing mechanism, such as promoter methylation. Methylation of the promoter region causes gene transcription silencing, which can be reversed by 5-aza-2′-deoxycytidine. We found that Su(Fu) became detectable in NCI-H209 cells in the presence of 5-aza-2′-deoxycytidine for 6–8 days (Fig. 3B), confirming that the Su(Fu) gene was silenced through promoter methylation in these cells.

To demonstrate the tumor suppressing role of Su(Fu), we stably expressed wild type Su(Fu) in H209 cells using retrovirus-mediated gene transfer [29]. Protein expression was verified by western blot analysis (Fig. 3B). By comparison of the levels of PTCH1 and Gli1 transcripts using real-time PCR analysis [26], we found that stable expression of Su(Fu) caused dramatic reduction in hedgehog target genes GLi1 and PTCH1 (Fig. 3C), indicating that Su(Fu) is sufficient to inhibit hedgehog signaling in this cell line. To demonstrate the tumor suppressor activity of wild type Su(Fu), we performed colony formation analysis in Su(Fu) negative H209 cells. Cells with ectopic expression of wild type Su(Fu) or the control vector were selected with G418 for 2 weeks, and cell colonies were stained with violet blue. Expression of Su(Fu) caused reduction of both the colony number and the size (Fig. 3D), indicating that

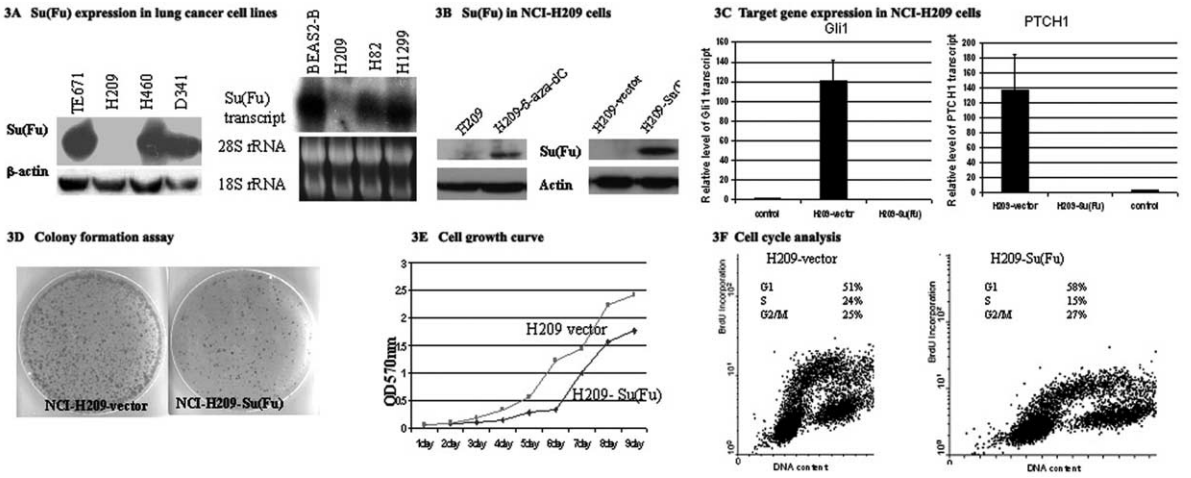


Fig. 3. Role of Su(Fu) in cancer cell lines Su(Fu) was detected by western blotting using Su(Fu) specific antibodies (see Section 2 for details). One of the eight lung cancer cell lines, NCI-H209, has no detectable Su(Fu) protein (A, also see Supplementary Fig. 1). Su(Fu) transcripts were undetectable in H209 cells by northern hybridization (A), indicating inactivation of the Su(Fu) gene. The inactivation of Su(Fu) was reversible in H209 cells in the presence of 5-aza-2′-deoxycytidine for over 6 days (B left), indicating an epigenetic mechanism of Su(Fu) silencing in these cells. Following expression of Su(Fu) in H209 cells, Su(Fu) protein was detected by western blotting (B right). Following stable expression of Su(Fu), levels of PTCH1 and Gli1 transcripts were detected by real-time PCR and found to be dramatically reduced (2C), indicating that Su(Fu) expression was sufficient to suppress the hedgehog signaling pathway. In contrast, expression of Su(Fu) has no effects on A549 cells (data not shown). Colony formation assay was performed to test the role wild type Su(Fu) on cell growth in H209 cells. Cells transfected with Su(Fu) formed small and few colonies (D). To confirm this result, the cell growth curve from Su(Fu) stably expressed H209 cell line was compared with that from the control cell line (E). Su(Fu) expression slowed cell growth. Furthermore, we performed BrdU labeling in these two cell lines (F). Following BrdU labeling for 30 min, around 24% of H209-vector cells were BrdU positive. In contrast, only 15% BrdU positive cells were observed in H209-Su(Fu) cells ($P < 0.02$), indicating that Su(Fu) inhibits cell growth and DNA synthesis in H209 cells.

ectopic expression of Su(Fu) is sufficient to suppress cell proliferation of these tumor cells.

Next, we examined cell growth using MTT assay, and found that H209-Su(Fu) cells grow slower than H209-vector cells, confirming that Su(Fu) indeed can suppress cell growth (Fig. 3E). The effect of Su(Fu) on DNA synthesis was assessed with BrdU labeling (Fig. 3F). In H209 cells, we found around 24% of cells are positive for BrdU after 30 min labeling with BrdU. In contrast, we only observed that 15% of cells with stable expression of Su(Fu) were BrdU positive. The difference is significant ($P < 0.02$). In contrast, Su(Fu) has no effects on DNA synthesis of A549 cells, which have no activated hedgehog signaling (data not shown here). These data indicate that wild type Su(Fu) can inhibit DNA synthesis and cell growth in lung cancer cells.

Taken together, our findings indicate that activation of the hedgehog signaling pathway, which occurs only in 15 out of 172 lung cancers (8.7%), is not a very common event in lung cancer although sonic hedgehog is frequently over-expressed. Our data suggest that Shh over-expression or loss of Su(Fu) may be responsible for hedgehog signaling activation in a small subset of lung cancers. Using a cell line with no detectable Su(Fu) protein, we demonstrated that expression of Su(Fu) is sufficient to inhibit the hedgehog signaling, leading to reduced DNA synthesis and inhibited cell growth. Thus, Su(Fu) inactivation appears to be another mechanism by which the hedgehog pathway is activated in subset of human lung cancer.

## 4. Discussion

Our data indicate that only a small proportion of lung tumors have expression of two hedgehog target genes (8.7%). We further find that loss of Su(Fu) was observed in 2 of the 16 tumors and one cell line NCI-H209, in which elevated hedgehog target genes were detected. The role of Su(Fu) is demonstrated in NCI-H209 cells. Thus, our data provide evidence that hedgehog signaling activation occurs in only a small percentage of lung cancer in which hedgehog signaling may be involved in cancer cell proliferation. Our studies further indicate that activation of the hedgehog pathway can be achieved by either Shh over-expression or Su(Fu) inactivation in a subset of lung cancer.

While activation of hedgehog signaling occurs infrequently, Shh is frequently over-expressed in lung cancers (Tables 1 and 2). Although Shh is weakly detectable in an inflammatory lung tissue

(Supplementary Table 1), all three normal lung tissues had no detectable Shh, indicating that Shh expression may be a biomarker of abnormal lung pathology. Based on the fact that the target genes were not elevated in these tissues, it will be interesting to investigate the functions of Shh in preneoplastic lesions as well as in lung cancers. We speculate that Shh expression is induced during lung cancer development long before the induction of the target genes. An early report indicates that sonic hedgehog may be involved in generating progenitor cells of lung [17]. Further investigation of hedgehog expression in a large number of inflammatory lung tissues and other pathological conditions may provide additional clues of sonic hedgehog functions in lung tissues.

## Acknowledgements

This research was supported by grants from the NIH (R01-CA94160, JX), Department of Defense (DOD-PC030429, JX), the NIEHS (ES06676) and National Science Foundation of China (30228031, JX and HWZ).

## Supplementary Material

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.canlet.2005.11.036.

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**Carcinogenesis** vol.27 no.7 pp.1334–1340, 2006
doi:10.1093/carcin/bgi378
*Advance Access publication February 25, 2006*

# Activation of the hedgehog pathway in human hepatocellular carcinomas

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**Liver cancers, the majority of which are hepatocellular carcinomas (HCCs), rank as the fourth in cancer mortality worldwide and are the most rapidly increasing type of cancer in the United States. However, the molecular mechanisms underlying HCC development are not well understood. Activation of the hedgehog pathway is shown to be involved in several types of gastrointestinal cancers. Here, we provide evidence to indicate that hedgehog signaling activation occurs frequently in HCC. We detect expression of *Shh*, *PTCH1* and *Gli1* in 115 cases of HCC and in 44 liver tissues adjacent to the tumor. Expression of *Shh* is detectable in about 60% of HCCs examined. Consistent with this, hedgehog target genes *PTCH1* and *Gli1* are expressed in over 50% of the tumors, suggesting that the hedgehog pathway is frequently activated in HCCs. Of five cell lines screened, we found Hep3B, Huh7 and PLC/PRF/5 cells with detectable hedgehog target genes. Specific inhibition of hedgehog signaling in these three cell lines by smoothened (SMO) antagonist, KAAD-cyclopamine, or with Shh neutralizing antibodies decreases expression of hedgehog target genes, inhibits cell growth and results in apoptosis. In contrast, no effects are observed after these treatments in HCC36 and HepG2 cells, which do not have detectable hedgehog signaling. Thus, our data indicate that hedgehog signaling activation is an important event for development of human HCCs.**

**Abbreviations:** DMEM, Dulbecco-modified essential medium; FBS, fetal bovine serum; HCCs, hepatocellular carcinomas; MTT, 1-(4,5-Dimethylthiazol-2-yl)-3,5-diphenylformazan; PCR, polymerase chain reaction; RT–PCR, reverse transcription–polymerase chain reaction; SMO, smoothened; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

[†]These authors contributed equally to this work.

## Introduction

Liver cancer, with hepatocellular carcinoma (HCC) as the major tumor type, is a malignancy of worldwide significance (1–4). HCC ranks as the eighth cause of cancer-related death in American men with 14 000 deaths yearly and is the most rapidly increasing type of cancer in the United States (2). The medical oncology community is largely unprepared for this looming epidemic of HCC. Although the increase of HCC in the United States is correlated with the increasing prevalence of chronic infection with hepatitis C virus (HCV), the molecular understanding of HCC development remains elusive (2). A majority (70–85%) of patients present with advanced or unresectable disease, making the prognosis of HCC dismal, and systemic chemotherapy is quite ineffective in HCC treatment. The first essential step for development of effective therapeutic approaches is to identify specific signaling pathways involved in HCC.

The role of the hedgehog pathway in human cancers has been established through studies of basal cell nevus syndrome (BCNS) (5,6), a rare hereditary disorder with a high risk of basal cell carcinomas, and activation of the hedgehog pathway has been observed in other cancers such as prostate cancer and gastrointestinal cancers (7–17). Targeted inhibition of the hedgehog pathway results in growth inhibition in cancer cell lines with activated hedgehog signaling (10–17). The hedgehog pathway is essential for embryonic development, tissue polarity and cell differentiation (18). The hedgehog pathway is critical in the early development of the liver and contributes to differentiation between hepatic and pancreatic tissue formation, but the adult liver normally does not have detectable levels of hedgehog signaling (10,19). In this report, we characterize expression of sonic hedgehog and its target genes in 115 HCC specimens. The role of hedgehog signaling on cell growth is further demonstrated in five HCC cancer cell lines.

## Materials and methods

### *Tissue samples*

A total of 115 specimens of HCC tissues were used. Of these, 14 specimens were received as discarded materials from General Surgery of the Shan Dong Qi Lu Hospital, Jinan, China. Pathology reports and H&E stained sections of each specimen were reviewed to determine the nature of the disease and the tumor histology. The remaining 101 HCC specimens were from Sun Yat-Sen University. Forty-four liver tissues adjacent to the tumor were also included in this study. None of the patients had received chemotherapy or radiation therapy prior to specimen collection.

### *In situ hybridization*

*In situ* hybridization was performed according to the manufacture's instructions (Roche Molecular Biochemicals, Indianapolis, IN) and our published protocol

(16,17). In brief, tissues were fixed with 4% paraformaldehyde in phosphate buffered saline (PBS) and embedded with paraffin. Then 6 μm thick tissue sections were mounted onto Poly-L-Lysine slides. Samples were treated with proteinase K (20 μg/ml) at 37°C for 15 min, refixed in 4% paraformaldehyde and hybridized overnight with a digoxigenin-labeled RNA probe (at a final concentration of 1 μg/ml). The hybridized RNA was detected by alkaline phosphatase-conjugated anti-digoxigenin antibodies (Roche Molecular Biochemicals, Indianapolis, IN), which catalyzed a color reaction with the substrate NBT/BCIP (Roche Molecular Biochemicals). Blue signal indicated positive hybridization. We regarded tissues without blue signals as negative. As negative controls, sense probes were used in the hybridization and no signals were observed. *In situ* hybridizations were repeated at least twice for each tissue sample with similar results.

*RNA isolation and quantitative RT–PCR*

Total RNA of cells was extracted using a RNA extraction kit from Promega according to the manufacturer (Promega, Madison, WI), and quantitative PCR analyses were performed according to a previously published procedure (17,20). Triplicate $C_T$ values were analyzed in Microsoft Excel using the comparative $C_T(\Delta\Delta C_T)$ method as described by the manufacturer (Applied Biosystems, Foster City, CA). The amount of target ($2^{-\Delta\Delta CT}$) was obtained by normalization to an endogenous reference (18S RNA) and relative to a calibrator. We used the following primers for RT–PCR of *Shh*: forward primer—5′-ACCGAGGGCTGGGACGAAGA-3′; reverse primer—5′-ATTTGGCCGCCACCGAGTT -3′

*Cell culture, transfection and drug treatment*

HCC cell lines [Hep3B, HepG2, HCC36, PLC/PRF/5 (as PLC throughout this manuscript) and Huh7] were generously provided by Drs Chiaho Shih, Tien Ko and Kui Li at UTMB. All cells were cultured in Dulbecco-modified essential medium (DMEM) with 10% FBS and antibiotics. Cells were treated with 2 μM KAAD-cyclopamine, a specific antagonist of smoothened (SMO) (21) (dissolved in DMSO as 5 mM stock solution, Cat# K171000 from Toronto Research Chemicals, Canada), in 0.5% FBS in DMEM for indicated time mentioned in the figure legends. Previously, we performed toxicity assay with KAAD-cyclopamine in GI cancer cells and found that 10 μM of KAAD-cyclopamine can lead to non-specific toxicity (16). In fact, 5 or 10 μM KAAD-cyclopamine was quite toxic to cells regardless of hedgehog signaling status (our unpublished observation), and was, thus, not used in this study. Tomatidine (2 μM in 0.5% FBS DMEM, Sigma Cat# T2909), a structurally similar compound with non-specific inhibition on hedgehog signaling, was used as a negative control. In addition, the specific inhibition of hedgehog signaling in HCC cells was achieved by addition of Shh neutralizing antibodies (1 μg/ml in 0.5% FBS DMEM, Cat# SC-9024, Santa Cruz Biotechnology, Santa Cruz, CA). Most cell lines were treated with KAAD-cyclopamine (2μM) or Shh antibodies (1 μg/ml) in 0.5% FBS in DMEM medium for an indicated time (see figure legends for details). However, for Hep3B cells, we used 2% FBS in DMEM because Hep3B cells cannot grow in 0.5% FBS DMEM medium. Transient transfection of Gli1 in HCC cells was performed using LipofectAmine according to manufacturer's recommendation (Plasmid:LipofectAmine = 1:2.5). Cells with ectopic expression of Gli1 were subjected to drug treatment and to TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling) assay.

*Cell viability and TUNEL assays*

For cell viability analysis, we used two methods: Trypan blue analysis and MTT assay. Trypan blue analysis was performed according to a procedure from the manufacturer (Invitrogen, CA) (22). The percentage of trypan blue positive cells (dead cells) was calculated under a microscope and triplicates of samples for each treatment were used. The experiment was repeated three times. MTT assay was performed using a previously published procedure (22). In brief, triplicates of samples for each treatment were used in a 96-well format. Twenty microliters of MTT (10 mg/ml in PBS) was added to each well (containing 100 μl cultured medium, 0.5% FBS DMEM in this study). Three hours later, medium was aspirated, and 100 μl of a mixture of isopropanol and DMSO (9:1) added into each well. Thirty minutes later, the 570 nm absorbance was measured with a microplate reader from Molecular Devices Co Sunnyvale, CA. BrdU labeling was for 1 h and immunofluorescent staining of BrdU was performed as reported previously (23). TUNEL assay was performed using a kit from Roche Biochemicals according to a published procedure (24). In brief, cells were fixed with 4% paraformaldehyde at room temperature for 1 h and permeated with 0.1% Triton X-100, 0.1% sodium citrate (freshly prepared) on ice for 2 min. After washing with PBS, each sample was incubated with 50 μl of TUNEL reaction mixture at 37°C for 30 min. TUNEL label solution (without enzyme) was used as a negative control. TUNEL positive cells were counted under a fluorescent microscope. The counting was repeated three times, and the percentage from each counting was calculated.

*Statistical analysis*

Statistical analysis was performed by Binomial proportions analysis. The association of mRNA transcript expression with various clinicopathological parameters was also analyzed; a $P$-value $< 0.05$ was considered to be statistically significant.

## Results

### *Expression of PTCH1 and Gli1 in primary HCC*

In order to assess hedgehog signaling activation in HCC, we assayed *PTCH1* and *Gli1* expression in 115 cases of HCC specimens. As the target genes of the hedgehog pathway, expression of *PTCH1* and *Gli1* transcripts indicate hedgehog signaling activation (25,26). Primarily, we used *in situ* hybridization to assess hedgehog signaling activation in our collected tissues ($n = 115$), which was further confirmed in selected specimens by real-time PCR. The results are summarized in Table I.

For *in situ* hybridization analysis, blue signal was regarded as detectable expression of the target. Tissues without blue signals were regarded as negative for the target. Using *in situ* hybridization, 79 of 110 (70%) tumor specimens had detectable expression of *Gli1* (representative images are shown in Figure 1A, and summarized in Table I, with additional images and data provided in Supplementary Table 1 and Supplementary Figures 1–6), indicating that *Gli1* expression is detectable in many HCCs. The sense probe gave no detectable signals (Figure 1A), confirming the specificity of *in situ* hybridization in our experiments. In most cases, *Gli1* expression was detectable in the tumor nest, not in the adjacent liver tissue (Figure 1A; Supplementary Figure 1 and Table 1) or in the stroma (arrows in Figure 1A).

In comparison with the *Gli1* transcript, the *in situ* hybridization signal of *PTCH1* was generally less intense (Figure 1B and Supplementary Figures 1–6), but 56% (60 of 107) of HCC specimens were positive for *PTCH1* transcript. We found a total of 51 tumors (out of 98 informative HCCs) (52%) with detectable expression of both *Gli1* and *PTCH1* (Table I, Supplementary Table 1), which suggests activated hedgehog signaling in these specimens. Our analysis indicates that activation of hedgehog signaling (as indicated by expression of both *Gli1* and *PTCH1* transcripts) occurs more frequently in HCC than in the adjacent liver tissue (Table I, Supplementary Table 1 and Supplementary Figure 1). There are several cases in which only *Gli1* or *PTCH1* was expressed (Supplementary Table 1), suggesting that expression of *Gli1* and *PTCH1* may be differentially regulated. Further analysis of our data did not reveal association of the hedgehog signaling activation with tumor size or tumor differentiation (Table I). Tumors with hepatocirrhosis were not significantly different from tumors without hepatocirrhosis in the expression of *Gli1* and *PTCH1* (Table I).

*In situ* hybridization data was further confirmed by real-time PCR in several tumor specimens in which 70% of the tissue mass was actually tumor tissue (Figure 1C and D). Consistent with *in situ* hybridization, expression of *Gli1* and *PTCH1* were detectable in the tumor, not in the adjacent liver tissue in most cases (will be discussed later in the Discussion). Our data indicate that expression of *Gli1* and *PTCH1* in the tumor was 3- to 30-fold higher than that in adjacent liver tissues (Figure 1C and D). The real-time PCR analyses further confirmed that activation of the hedgehog pathway is a common event in HCC.

**Table I.** Detection of *Shh*, *PTCH1* and *Gli1* expression in HCC and in adjacent liver tissue by *in situ* hybridization

| | *Shh* | | | Hedgehog pathway activation | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | *PTCH1* | | *Gli1* | | Pathway activation | | |
| | pos | neg | *P*-value | pos | neg | pos | neg | pos | neg | *P*-value |
| HCC | 64/108 | 44/108 | <0.01* | 60/107 | 47/107 | 79/110 | 31/110 | 51/98 | 47/98 | <0.01* |
| Adjacent tissues | 5/41 | 36/41 | | 18/43 | 25/43 | 15/44 | 29/44 | 9/43 | 34/43 | |
| Tumor size | | | | | | | | | | |
| Small (<3 cm) | 16/31 | 15/31 | 0.316 | 17/31 | 14/31 | 25/32 | 7/32 | 16/31 | 15/31 | 0.896 |
| Large (>3 cm) | 46/74 | 28/74 | | 42/74 | 32/74 | 52/75 | 23/75 | 35/66 | 31/66 | |
| Tumor differentiation | | | | | | | | | | |
| Well | 34/52 | 18/52 | 0.107 | 30/51 | 21/51 | 43/52 | 9/52 | 29/51 | 22/51 | 0.264 |
| Mod-poor | 20/41 | 21/41 | | 22/41 | 19/41 | 32/43 | 11/43 | 19/42 | 23/42 | |
| Sex | | | | | | | | | | |
| Male | 47/81 | 34/81 | 0.651 | 43/81 | 38/81 | 58/83 | 25/83 | 35/72 | 37/72 | 0.258 |
| Female | 17/27 | 10/27 | | 17/26 | 9/26 | 21/27 | 6/27 | 16/26 | 10/26 | |
| Hepatocirrhosis | | | | | | | | | | |
| + | 14/19 | 5/19 | 0.163 | 14/20 | 6/20 | 14/20 | 6/20 | 11/17 | 6/17 | 0.251 |
| – | 49/87 | 38/87 | | 43/83 | 40/83 | 63/87 | 24/87 | 39/79 | 40/79 | |

Statistical analysis was performed by Binomial proportions analysis. A $P$-value < 0.05 was considered to be statistically significant. The association of mRNA transcript expression with various clinicopathological parameters was also analyzed. Statistically significant difference was indicated by asterisk (*).

pos, positive signal; neg, negative signal; well, well-differentiated tumors; mod-poor, moderately to poorly differentiated tumors. Elevated expression of at least two hedgehog target genes was regarded as being positive (pos) in activation of the hedgehog pathway, whereas elevated expression of one hedgehog target gene was regarded as being negative (neg) in hedgehog signaling activation.

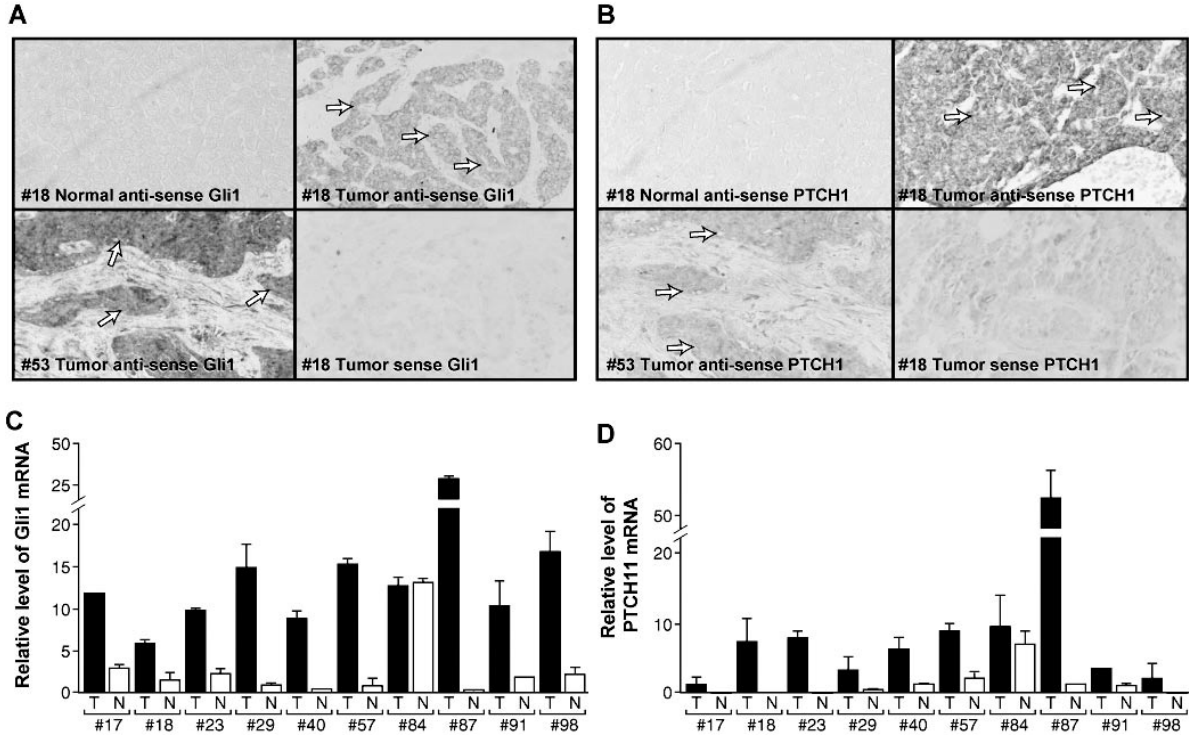


**Fig. 1.** Detection of *Gli1*, *PTCH1* expression in primary HCCs. *In situ* hybridization detection of *Gli1* (**A**) and *PTCH1* (**B**) transcripts in HCCs was performed as reported previously. Positive signals (dark grey staining) were observed in the tumor ('Tumor', tumor nests indicated by arrows), not in the stroma surrounding the tumor nests or in the liver tissue adjacent to the tumor ('Normal'). The sense probes did not give any positive signals (A and B), confirming the specificity of our *in situ* hybridization. Additional pictures have been included in the Supplementary Figures. Expression of *Gli1* and *PTCH1* was further confirmed by real-time PCR analysis done in triplicate (**C** and **D**) in selected tumor specimens in which 70% of the tissue mass was tumor tissue. Expression of *Gli1* (C) and *PTCH1* (D) from the tumor (T) was 3- to 30-fold higher than that from the adjacent liver tissue (N). Data indicates values relative to 18S RNA and to a calibrator. The data from this analysis are consistent with those from *in situ* hybridization analysis.

### *Expression of Shh in HCCs*

To investigate if *Shh* is associated with hedgehog signaling activation in HCCs, *Shh* expression was first detected by *in situ* hybridization. We detected *Shh* transcripts in 64 of 108 HCC specimens, but not in the majority of liver tissues adjacent to the tumor (Figure 2A, Table I and Supplementary Figures 1, 4–6). *Shh* transcript was only detectable in the tumor nests, not in the stroma (dark grey signals in Figure 2A), suggesting that

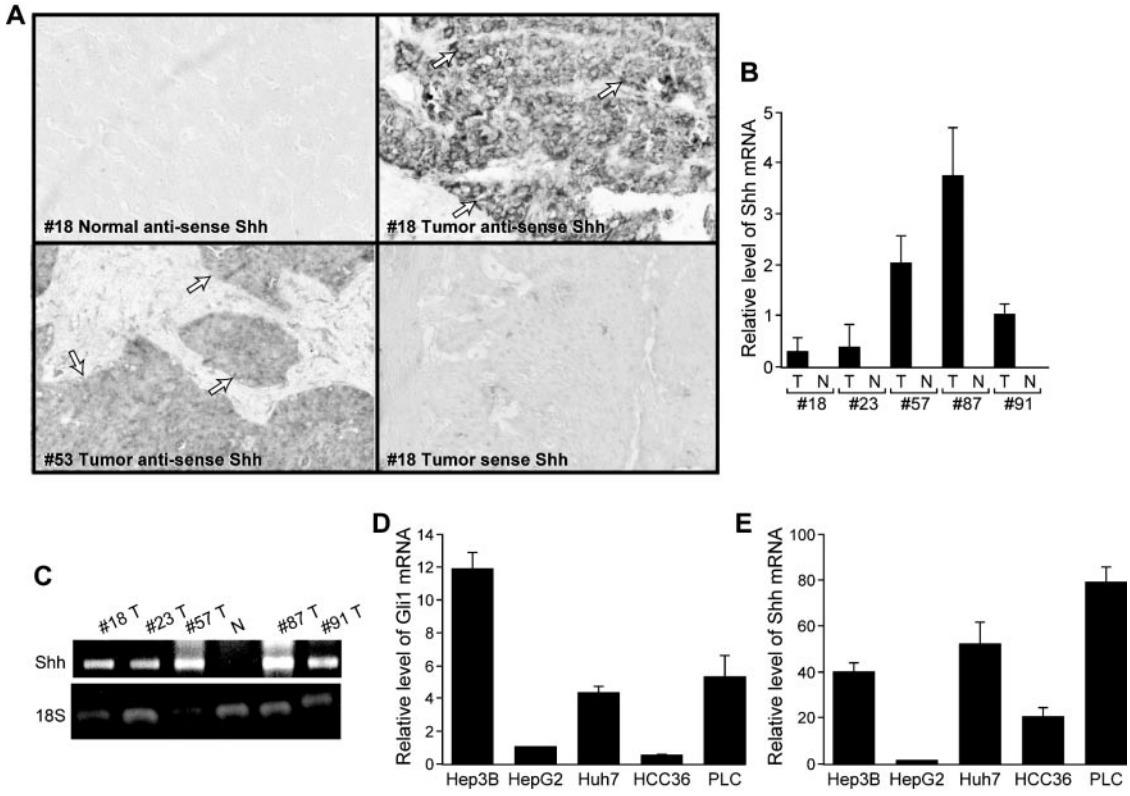


**Fig. 2.** Detection of *Shh* expression in HCCs. *In situ* hybridization (**A**), real-time PCR (**B**) and regular RT–PCR (**C**) were used to detect *Shh* transcript. *Shh* transcript (dark grey signals in A) resided in the tumor ('Tumor', tumor nests indicated by black arrows), not the stromal or adjacent liver tissue ('Normal') (A), suggesting that the tumor tissue is the major source for *Shh* expression. To confirm our *in situ* hybridization results, we used real-time PCR to detect *Shh* expression (B), which was further confirmed by RT–PCR (C). Shh transcripts were detected only in the tumor (T), not in the adjacent liver tissue (N). Tumors with detectable *Gli1* and *PTCH1* transcripts all had detectable *Shh*, suggesting a major role of *Shh* for activation of the hedgehog pathway in HCCs. Additional real-time PCR experiments showed a relatively high level of *Gli1* (**D**), *PTCH1* (not shown here) and *Shh* (**E**) in three HCC cell lines: Hep3B, Huh7 and PLC. Data indicates values relative to 18S RNA and to a calibrator.

cancer cells are the major source of *Shh* expression. Almost all tumors with detectable *Gli1* and *PTCH1* expression had detectable *Shh* transcript (Figures 1 and 2, Supplementary Table 1, Supplementary Figures 5 and 6). *Shh* expression in the tumor was further confirmed by real-time PCR and regular RT–PCR (Figure 2B and C). Thus, it appears that *Shh* induction may be the trigger for activated hedgehog signaling in HCCs. In support of this hypothesis, we detected expression of *Shh* in all three HCC cell lines with detectable transcript of *Gli1* (Figure 2D and E).

*Targeted inhibition of hedgehog signaling in HCC cells*

SMO is the major signal transducer of the hedgehog pathway; thus cancer cells with activated hedgehog signaling through *Shh* expression should be sensitive to treatment with the SMO antagonist, KAAD-cyclopamine (Toronto Research Chemicals, Cat# K171000, Toronto, Canada) (21). First, we screened HCC cell lines for hedgehog signaling activation by real-time PCR detection of *Gli1* and *PTCH1* and found that hedgehog signaling pathway was activated in Hep3B, PLC and Huh7 cells but not in HepG2 and HCC36 cells (Figure 2D shows the level of *Gli1* transcript). Addition of KAAD-cyclopamine (2 μM) greatly decreased the level of *Gli1* transcript in three cell lines (Hep3B, PLC and Huh7) (Figure 3A), whereas no changes on *Shh* expression were observed (Supplementary Figure 7). The closely related compound tomatidine, which does not affect SMO signaling and thus served as a negative control, had little discernible effect on hedgehog target genes. This data indicates specific inhibition of the hedgehog pathway by KAAD-cyclopamine in these cells.

As a result of inhibited hedgehog signaling by KAAD-cyclopamine treatment, we observed an inhibition on cell growth of Huh7 cells, but not on that of HepG2 cells (Figure 3B and C). The specificity of hedgehog signaling inhibition was further demonstrated using Shh neutralizing antibodies (Figure 3B and C). We found that addition of Shh antibodies at a concentration of 1 μg/ml reduced cell growth of Huh7 cells but had no effect on HepG2 cells (Figure 3B and C). Further analysis indicates that BrdU incorporation was also reduced after treatment with KAAD-cyclopamine in Huh7 cells (see Supplementary Figure 8).

Following treatment with KAAD-cyclopamine or Shh antibodies, we found that PLC cells underwent apoptosis whereas no apoptosis was observed in HepG2 cells (Figure 4A shows data from KAAD-cyclopamine treatment). Data from TUNEL assay was confirmed by Trypan blue staining (data not shown here). The percentage of apoptotic cells varied from cell line to cell line, with PLC being the most sensitive cell line (over 20% TUNEL positive cells after KAAD-cyclopamine treatment for 8 h, Figure 4B). Similar data were also observed after Shh antibody treatment (data not shown here). These data demonstrate that the HCC cells with activated hedgehog signaling are sensitive to targeted inhibition of the hedgehog pathway, whereas other HCC

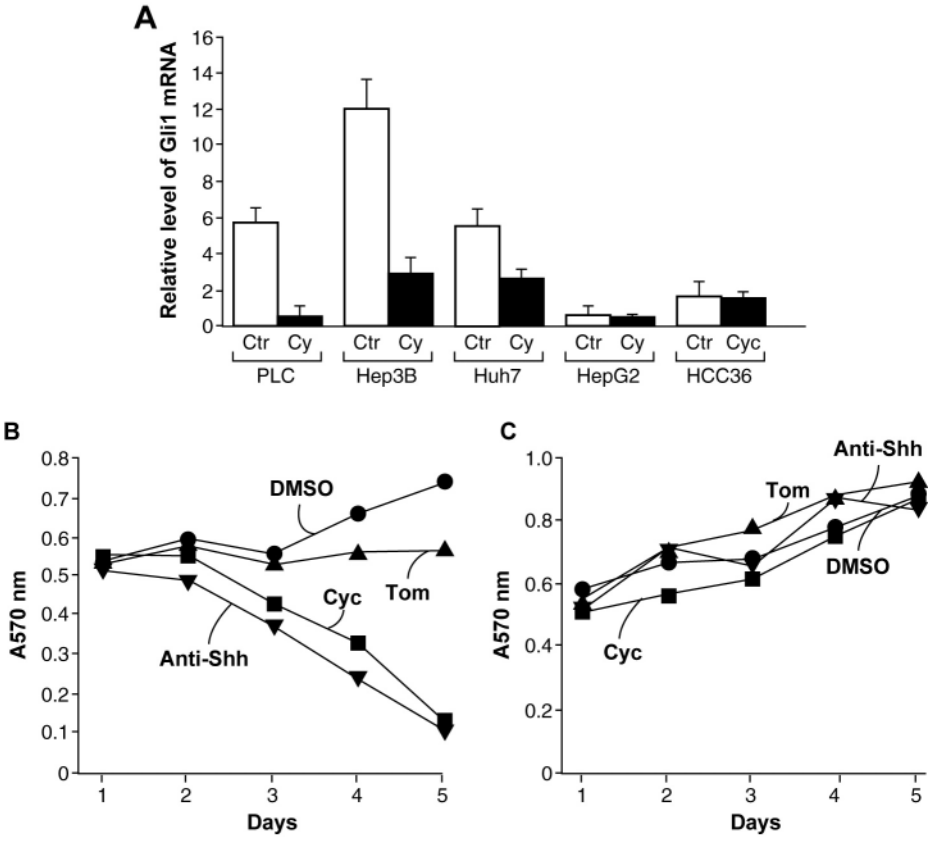


**Fig. 3.** Hedgehog signaling and growth of HCC cells. Real-time PCR data of *Gli1* transcript shows that in the presence of 2 μM KAAD-cyclopamine (**A**) or 1 μg/ml Shh neutralizing antibodies (data not shown here) for 12 h (see Materials and methods for details on drug-treatment conditions), the level of hedgehog target gene *Gli1* was decreased in the three cell lines with activated hedgehog signaling (PLC, Hep3B and Huh7). In contrast, no effects were observed in HCC36 and HepG2 cells, in which hedgehog signaling is not activated. Cell growth of Huh7 (**B**) and HepG2 (**C**) cell lines were examined by MTT assay. Huh7 cells were inhibited by 2 μM KAAD-cyclopamine (Cat# K317000, Toronto Research Chemicals) or 1 μg/ml Shh neutralizing antibodies (Cat# 9024, Santa Cruz Biotechnology) (Figure 2B). This inhibition was specific because addition of tomatidine, a structurally similar but non-specific compound for hedgehog signaling, did not affect cell growth. In contrast, cell growth of HepG2 was not affected by KAAD-cyclopamine (2 μM) or Shh neutralizing antibodies (1 μg/ml) (C), confirming the specific growth inhibition of HCC cells through targeted inactivation of hedgehog signaling.

cells (without activated hedgehog signaling) are resistant to these treatments.

Because KAAD-cyclopamine and Shh antibodies only affect signaling upstream of SMO, we hypothesize that cells with ectopic expression of the downstream effector Gli1 may prevent KAAD-cyclopamine-mediated apoptosis if these treatments are specific to the hedgehog pathway. In Huh7 cells, we transiently expressed Gli1 under the control of the CMV promoter (pLNCX vector) (23). After KAAD-cyclopamine treatment, we found that all Gli1-expressing cells ($n = 500$) were negative for TUNEL, demonstrating the specificity of KAAD-cyclopamine. Similarly, Gli1-expressing Huh7 cells were resistant to Shh antibody treatment (data not shown). This study also suggests that downregulation of *Gli1* may be an important mechanism by which targeted inhibition of hedgehog signaling mediates apoptosis in HCC cells.

Taken together, our findings indicate that activation of the hedgehog pathway is quite common in liver cancers. Expression of *Shh* and its target genes, *Gli1* and *PTCH1*, is more frequent in the tumor than in the adjacent liver tissue. This activation of hedgehog signaling is not associated with other clinicopathological parameters of the tumor. HCC cells with activation of the hedgehog pathway are sensitive to targeted inhibition of hedgehog signaling. These data support our hypothesis that activation of the hedgehog pathway is an important event in the development of HCC.

## Discussion

### *Hedgehog signaling in liver cancer*

Over 500 000 new cases of liver cancers are reported each year worldwide; most of them are HCCs. Most of HCC patients (70–80%) are diagnosed late in the progression of the disease and cannot be effectively treated. Understanding the molecular mechanisms underlying liver cancer development is an essential first step in early diagnosis of liver cancer. In this report, we present strong evidence to indicate that the hedgehog pathway is frequently activated in liver cancers. Our data further indicate that induced expression of *Shh* may be the major trigger for activated hedgehog signaling in HCCs. How was *Shh* expression induced in HCC? Our preliminary data indicate that the Shh promoter activity is high in Huh7 cells but low in HepG2 cells (our unpublished observation), suggesting that transcriptional upregulation of the *Shh* gene may be the major mechanism for induced expression of *Shh*.

Since hedgehog signaling is frequently activated in HCCs, markers for hedgehog signaling activation, including *Shh*, *PTCH1* and *Gli1*, may be useful for diagnosis of liver cancers. In most cases, *Gli1* and *PTCH1* were expressed in the tumor,

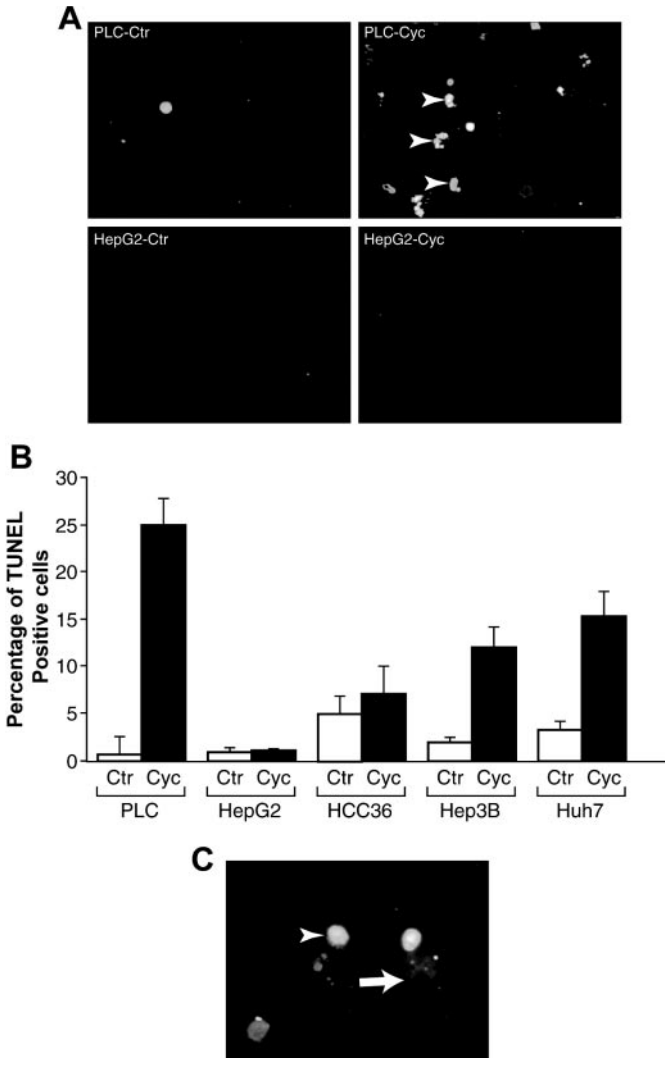


**Fig. 4.** Targeted inhibition of hedgehog signaling induces apoptosis in hepatocellular carcinoma cells. TUNEL assays (**A**) for detection of apoptosis were measured after treatment with 2 μM KAAD-cyclopamine (Cat# K317000, Toronto Research Chemicals) in PLC (8 h) and in HepG2 (36 h). During treatment, we used 0.5% FBS in DMEM for cell culture. TUNEL positive cells (light grey, indicated by arrowheads) were observed in PLC cells after KAAD-cyclopamine treatment. The percentages of TUNEL positive cells in all five cell lines are quantified in **B.** Please note that different cell lines have different sensitivities to KAAD-cyclopamine. Whereas treatment of 2 μM KAAD-cyclopamine for 8 h was sufficient to induce cell death in PLC cells, a longer treatment was needed for Hep3B (48 h) and Huh7 cells (36 h) (also see Materials and methods for drug treatment). TUNEL positive cells ($n = 500$) were counted under a fluorescent microscope, and the experiment was repeated three times with similar results. TUNEL assays following ectopic Gli1 expression and KAAD-cyclopamine treatment (**C**) demonstrated that cells positive for ectopic Gli1 expression [dark grey staining, detection by c-myc tag as reported previously (27)] were TUNEL negative. Gli1 was expressed in Huh7 cells under the control of the CMV promoter. Transfected cells were treated with 2 μM KAAD-cyclopamine or 2 μM tomatidine in 0.5% FBS in DMEM for 48 h. We found that Gli1-expressing cells ($n = 500$) were all TUNEL negative.

not in the liver tissues adjacent to the tumor. However, in nine cases, we detected expression of *Gli1* and *PTCH1* in both the tumor and the adjacent liver tissues, which were confirmed by real-time PCR in one case (#84) (see Supplementary Table 1 for details). Further analysis indicated that tissue abnormalities were present in these adjacent liver tissues with expression of *Gli1* and *PTCH1*, ranging from small cell dysplasia, dysplastic nodules to microscopic HCCs. In contrast, a non-cancerous liver tissue (as shown in supplementary Figures 2E, 3E and 4E) did not have any detectable expression of *Shh*, *PTCH1* and *Gli1*. Thus, it appears that hedgehog signaling activation occurs in early lesions of HCCs. Further studies of hedgehog signaling in different stages of HCCs, particularly early stages, will establish the basis for early diagnosis of HCC through detection of *Gli1*, *PTCH1* and *Shh*.

Another important pathway involved in HCC is the Wnt pathway via mutations of β-catenin or axin (28–31). We have investigated the association of hedgehog signaling with the Wnt pathway in liver cancer. We detected β-catenin protein localization by immunohistochemistry in tumors with activated hedgehog signaling. Only 1 in 20 tumors with hedgehog signaling activation had nuclear β-catenin, a major indicator for the canonical Wnt signaling, suggesting that hedgehog signaling activation may be a distinct abnormality from β-catenin activation in HCCs.

### *Therapeutic perspective of liver cancer through targeted inhibition of the hedgehog pathway*

Our studies also indicate that targeted inhibition of hedgehog signaling may be effective in treatment of HCCs. We demonstrate in this report that SMO antagonist, KAAD-cyclopamine, or Shh neutralizing antibodies specifically induce apoptosis in HCC cells with activated hedgehog signaling. The hedgehog pathway is not activated in HepG2 cells, and these cells are not sensitive to these reagents. In our studies, variable sensitivities were observed in different cell lines. For PLC cells, treatment with 2 μM KAAD-cyclopamine for 8 h caused apoptosis in many cells. In contrast, a similar rate of cell death was observed in Huh7 cells after treatment (2 μM KAAD-cyclopamine) for 36 h. This difference may be due to other genetic alterations in different cell lines. Further understanding of the molecular basis for cell sensitivity to KAAD-cyclopamine will help us to design better ways to treat HCC in the future. Thus, it may be possible in the future to treat the subsets of liver cancer with hedgehog signaling inhibitors (e.g. KAAD-cyclopamine).

While this manuscript is being reviewed, two other groups have reported similar data on hedgehog signaling in HCCs (32,33).

## Supplementary material

Supplementary material is available at: http://www.carcin.oxfordjournals.org/

## Acknowledgements

This research was supported by grants from the NIH (R01-CA94160), Department of Defense (DOD-PC030429), The NIEHS (ES06676) and National Science Foundation of China (30228031). We thank Drs Chiaho Shih, Tien Ko and Kui Li for providing cell lines for this study and Huiping Guo for technical support in real-time PCR analysis and Karen Martin for help with the manuscript. Funding to pay the Open Access publication charges for this article was provided by National Cancer Institute (R01-CA94160).

*Conflict of Interest Statement*: None declared.

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*Received September 1, 2005; revised February 4, 2006; accepted February 19, 2006*